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Discontinuous Electrophoresis

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Introduction

Electrophoresis is one of the most powerful tools in the arsenal of separation scientists. It is commonly employed in the field of biochemistry, where separation of complex mixtures of proteins or nucleic acids is a continuing challenge. Numerous variants of electrophoresis have been described with the goal of optimizing the speed and effectiveness of the separations. One important electrophoretic variable is the separation matrix. It provides the retarding forces, or sieving qualities, that counter the electrophoretic transport. These forces can ultimately effect the separation and can be altered by the matrix type or concentration. Cross-linked or linear forms of polymers such as agarose or acrylamide are common choices. Different formats for the electrophoresis medium can also have dramatic effects on the resolution and separation time. This is exemplified by recent uses of microcapillary formats which greatly speed up separations. Another component that dictates the speed and resolution of electrophoretic separations is the charge-carrying buffer ion. The buffer is a universal component of electrophoresis, independent of gel constitution or format. This component is often overlooked, though attention to this aspect can be beneficial in developing electrophoresis-based separation techniques.

Proper buffer selection offers several practical advantages, including optimum separation times, increased band concentration and reduced effects of diffusion. The resolution of closely migrating species results from the proper choice of the pH, concentration, and type of buffer ion. These physical characteristics define the conductivity of the electrophoretic medium and affect the transport of the molecules to be separated. Inorganic ions, such as chloride anion, have high conductivities in comparison to the ionized form of weak acids and bases. Such high mobility ions offer little advantage when used for the electrophoretic separation of large, less mobile biomolecules but slower-moving ions, such as those of weak acids or weak bases, are more useful choices. These not only buffer the pH but, due to the slower mobility of these ions, lead to better separation of charged macromolecules.

Zonal electrophoresis utilizes a single buffer in the gel and reservoirs. An alternative to the continuous buffer, zonal separations is a discontinuous system where multiple ionic components are used. The presence of multiple ionic components in electrophoresis leads to discontinuities in the voltage gradient, pH and ionic strength due to the different physical mobilities of the ions involved. These different mobilities lead to the formation of discrete zones of ions that, under equilibrium conditions, travel at a constant rate in an applied electric field. Adjustment of these mobilities involves alteration of the ion concentration and potential gradient of the zone. Sharp boundaries can exist between these zones, with the ionic concentration being dictated by the Kohlrausch regulating function. The technique is similar or identical to a number of electrophoresis techniques that are known as discontinuous multiphasic, multizonal,

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displacement, isotachophoresis and moving boundary electrophoresis. The primary advantages of discontinuous buffer systems over continuous buffer, zonal separation are their ability to concentrate dilute samples, enhance resolution between closely migrating species and provide defined reference fronts.

Implementation

Most of the electrophoretic techniques that exploit the differential migration of ions in an electric field trace their origins to the work of Kohlrausch. In 1897, Kohlrausch presented the equations that describe ionic migration in an electric field. In later decades, Tiselius furthered electrophoretic techniques and accomplished the separation of serum proteins using moving boundary electrophoresis. In 1958, Poulik published the use of the discontinuous buffer technique for the separation of proteins in a starch gel and, a few years later, Ornstein and Davis described the theory of discontinuous buffer systems for the separation of serum proteins in polyacrylamide gels. The Ornstein and Davis 'disc' electrophoresis system concentrated the sample by placing it between buffers of different mobility and then performed the separation using zone electrophoresis. In 1965, Richards applied the technique for the separation of nucleic acids. Since this period, discontinuous buffer systems, or similar techniques, have seen consistent usage in protein separations. Theoretical treatments that aid in the design of appropriate buffer systems have been extended. This has led to the description of various buffer systems for the separation of both acidic and basic proteins and the development of 'spacer' ions for resolving closely migrating proteins. The application to nucleic acid separations has been sporadic. This is perhaps due to the lower reliance on charge differences for the separation of nucleic acids. In contrast to proteins, nucleic acids have a near constant mass-to-charge ratio giving them a common free electrophoretic mobility. Typically, zonal gel systems that fractionate solely on size are used. However, discontinuous buffer systems do offer advantages for certain nucleic acid separations.

In practice, a leading ion is chosen such that the mobility of this ion is greater than all others in the system. This ion is incorporated into the separation matrix (or in some applications, an anti-convective matrix). The trailing ion(s), with a mobility slower than the leading ion, is placed in the top buffer reservoir while a counterion, common to all zones, is placed in the bottom buffer reservoir. For open-faced gel systems, the samples are loaded directly on to the separation matrix, while for vertical slab systems the sample can be loaded on top of the gel. As the moving boundary is established, the sample will be concentrated in a very thin moving boundary. The analytes to be separated will stack themselves in the order of their ionic mobility and, in the case of discontinuous electrophoresis, may be further fractionated by the use of a sieving gel. The resultant bands are easily detected with conventional techniques, using stains or fluorescent or radioactive labels. A description of a typical experimental set-up, and the identity of the various ionic species for an anionic separation, is shown in **Figure 1**.

Only the concentration of the leading ion can be chosen freely. During electrophoresis, the ionic concentration and potential gradient of the trailing zones will be regulated to compensate for the lower mobility. This regulating function is known as the Kohlrausch regulating function. Under equilibrium conditions, the leading and trailing ions migrate at the same rate (isotachophoresis). This causes the ion concentration in a trailing zone to be lower, and the voltage gradient to be higher, than that in a leading

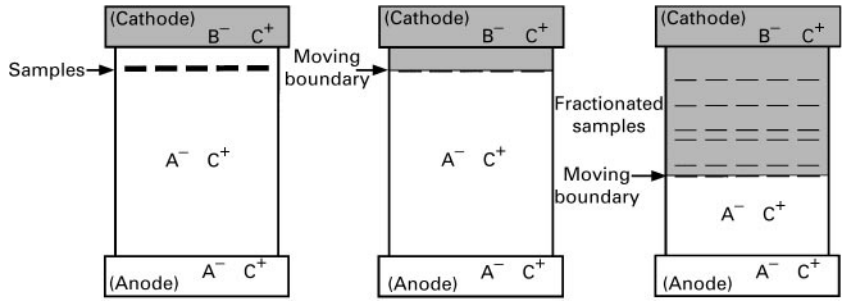

Figure 1 Pictorial description of the progress of an electrophoretic separation using a discontinuous buffer system. The initial set-up is shown on the left. A trailing anion, B^-, is placed in the cathodol buffer reservoir while the leading ion, A^-, is placed in the gel. This leading anion has a mobility that is greater than all other anions in the system. A common counterion, C^+, is used throughout the reservoirs and gel. The centre panel shows the sample being concentrated by the moving boundary created by the dissimilar anions. As this boundary traverses through the gel, the sample components, with mobilities that are slower than the trailing anion, will be fractionated in the trailing zone. Those ions with an intermediate mobility will be retained in the moving boundary. This is shown on the right.

zone. These differences compensate for the lower free mobility. The migration rate of the ions can be followed by observing the position of the boundary between the zones. This boundary is self-sharpening, as 'trailing ions', if present in the leading zone, will be in a low field region and slow down, while 'leading ions' in the trailing zone will be in a high field region and speed up. This boundary can be conveniently demarcated by incorporation of a low concentration of a dye with a mobility intermediate between the leading and trailing zone. Alternatively, conductivity or thermal changes can be monitored to detect the passing of the boundary. Multiple boundaries can exist with each one defining a distinct zone. Since the concentration of ions in the zone is regulated, the size of the zone will depend on the quantity of material and the concentration of the leading ion. Thus, dilute samples can be concentrated and different ions can be sorted based on their mobilities.

Selection of appropriate buffer systems usually requires some knowledge of the charge and mobility of the analytes to be separated. Many buffer systems, which span the entire range of pH values, have been described. These systems are especially useful for the separation of proteins that are charged under specific pH values. The mobilities of the charged analytes to be separated are also critical. For molecules that are subsequently to be fractionated within the trailing zone, the trailing ion should have a mobility that is similar to or intermediate between the ions to be separated. Tuning the mobility of the trailing ion to that of the analyte can minimize the conductance changes across the sample. This can lead to sharp band profiles and enhance the resolution of closely migrating species. Reference to previously tabulated buffer mobilities will aid in the selection of an appropriate buffer system. The development of new buffer systems can be accomplished empirically or through simple application of the equations describing discontinuous electrophoresis.

Advantages

The primary advantage of discontinuous buffer systems is the ability to concentrate the sample zone. The passage of the moving boundary has the effect of sweeping the sample into an extremely thin starting band. For analytical applications this can lead to reduced band widths and higher resolution separations. This feature has obvious advantages for the characterization of closely migrating species. For preparative applications, the result is the concentration of dilute samples. Another advantage of discontinuous buffer systems is their use as an analytical tool for defining relative mobilities. The mobility of the ion front is easily defined and can serve as a reference for defining relative mobilities. Furthermore, the mobility of the front is reproducible and independent of the gel matrix. These features can be convenient for analytical and forensic applications. Additionally, the mobility of sample ions can be altered by changing the mobility of ions in the trailing zone. This can lead to the tailoring of separations by defining the size range that can be fractionated. Sample ions that are not of interest can remain trapped in an ion front, allowing examination of particular ions. Finally, discontinuous buffer systems are compatible with virtually any gel format, as its use is independent of the gel matrix or the physical format of the gel. Since it alters only the buffer system, the only complication is selecting an effective buffer. Fortunately, the characteristics of many buffer systems have been described and designing new systems is a straightforward task, as described below.

Figure 2 exemplifies some of the features of discontinuous buffer systems for nucleic acid separations. Shown here is a portion of a DNA sequencing gel using a denaturing formate–glycine discontinuous buffer system. An intermediate zone, with a mobility between the formate leading ion and the glycine trailing ion, was inadvertently created from contaminating ions in the gel or sample loading buffer. For analytical application, care should be taken to ensure that extraneous ions are absent. However, the presence of this ion species demonstrates the effects of stacking limits on the size selection and band concentration of DNA sequencing products. DNA bands smaller than 106 bases are trapped in the first ion front while DNA sizes 115–166 are trapped in the second front. The DNA sizes that are trapped in the ion fronts define the stacking limits and cannot be separated because their mobility is between that of the migrating ion zones. Since the DNA is not freely electrophoresing, these stacking limits could be altered by changes to the gel matrix. The intermediate ion zone demonstrates other features. Its size is directly proportional to the amount of contaminating ion that is present. Larger amounts of contaminating ion would expand this zone. Additionally, the eight DNA bands in this zone are much sharper than the bands migrating behind the second front, even after the 30 cm migration distance. This is due to the concentrating effects of the leading ion front and the closely matched mobility between these DNA bands and the ions in the zone.

Theoretical Description

Calculation of the ion concentrations and basic characteristics of the leading and trailing ion zones are

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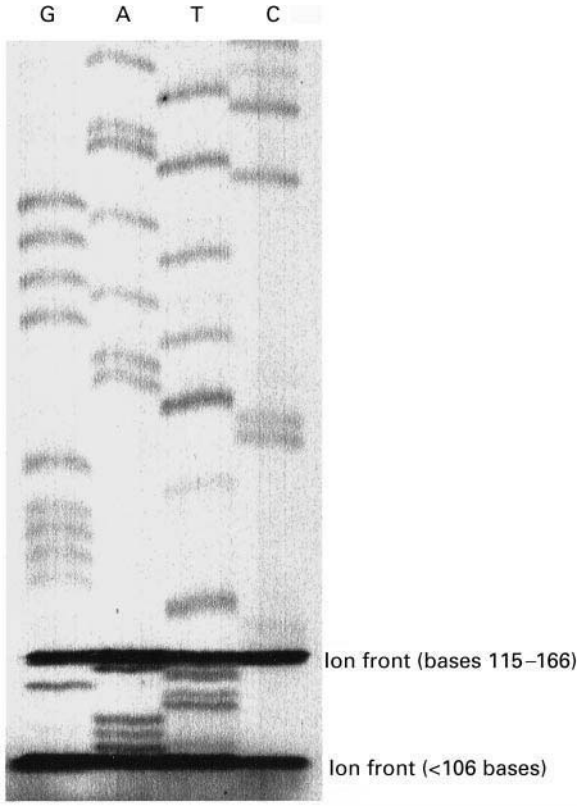

Figure 2 The differential effects of stacking limits on the migration of DNA sequencing products on a 6% polyacrylamide gel (containing 5% bis-acrylamide cross-linker) with a 50 mmoL L^{-1}, pH 9.0 formate leading ion, glycine trailing ion. The sequence between the two fronts, AAATTGTT, corresponds to fragment lengths of 107–114 bases; the sequence behind the front, CTGG, corresponds to fragment lengths of 167–170 bases. Fragment lengths between 115 and 166 bases are concentrated in the second front while fragment lengths < 107 bases are concentrated in the first front.

easily accomplished through use of a few simple equations. The starting point is the Kohlrausch regulating function (R) that describes the moving boundary condition:

$$\sum \frac{C}{m} = R_n \qquad [1]$$

where C is the concentration of the ion in zone n and m is the mobility of the ion. These values are signed according to the charge of the ion. For the set-up described in Figure 1, the regulating functions characterizing the two zones would be equal under moving boundary conditions and:

$$\frac{C_1^{A^-}}{m_{A^-}} + \frac{C_1^{C^+}}{m_{C^+}} = \frac{C_2^{B^-}}{m_{B^-}} + \frac{C_2^{C^+}}{m_{C^+}} \qquad [2]$$

where the concentration subscripts denote the zone. This relationship, along with the condition for electroneutrality:

$$C_1^{A^-} + C_1^{C^+} = 0 = C_2^{B^-} + C_2^{C^+} \qquad [3]$$

allows definition of the basic relationship between the concentration of ions in the different zones and their mobilities:

$$\frac{C_1^{A^-}}{C_2^{B^-}} = \frac{m_{A^-}(m_{B^-} - m_{C^+})}{m_{B^-}(m_{A^-} - m_{C^+})} \qquad [4]$$

This relationship can be defined for every boundary in a discontinuous electrophoresis system. The relationship is valid for strong electrolytes and weak electrolytes. For weak electrolytes, the concentration value represents the total of the ionized and un-ionized forms. The fraction of the trailing species that is ionized, $X(n)$, can be determined after calculation of the amount of counterion that crosses the boundary into the trailing zone. When using a weak electrolyte as the counterion, a final expression for the counterion concentration in the trailing zone is:

$$C_2^{C^+} = C_1^{C^+} + C_1^{A^-}\left(\frac{m_{C^+}}{m_{A^-}}\left(1 - \frac{(m_{A^-} + m_{C^+})}{(m_{C^+} + m_{B^-})}\right)\right) \qquad [5]$$

where the counterion concentrations are the total concentration (both ionized and un-ionized forms). The fraction ionized, $X(n)$, can then be calculated from the ion equilibrium constants of the trailing ion and counterion. The net mobility accounts for the actual transport of the trailing acid species and is defined as:

$$\text{net mobility}(n) = m_n X[n] \qquad [6]$$

The net mobility should be tuned closely to the sample's mobility. Sample mobilities faster than the trailing ion net mobility will be retained in the ion front. Details regarding these equations and more rigorous definitions can be found in numerous references.

A few characteristics of discontinuous buffer systems are apparent from examining the above equations. First, the equilibrium concentration of the trailing ion is independent of its initial concentration. It is determined by the free mobilities of the ions in the system and the concentration of the leading ion. Therefore, only the leading ion conditions can be chosen freely. Second, since the trailing ion has a slower mobility than that of the leading ion, the concentration of the trailing ion must be lower than that of the leading ion. This bears directly on the conductance and potential gradient of the trailing

zone. The zone conductance, κ, can be calculated from the ion concentrations, free mobilities and Faraday's constant (F). It can also be related to the field strength (volts (V) per unit length (l)) through Ohm's law, as shown (where i is the current and A the cross-sectional area of the gel):

$$\kappa = F\,[(\text{net mobility}(B^-))(C_2^{B^-}) + (\text{net mobility}(C^+))(C_2^{C^+})] = \frac{i}{A(V/l)} \qquad [7]$$

Since the ion concentrations and ion mobilities in the trailing zone are lower than that in the preceding zone, the potential gradient in a trailing zone will be higher than the leading zone. This is what allows the trailing ions to migrate at the same rate as the leading ions, despite their lower physical mobility. Tuning the ionic strength is critical for optimizing separations. It affects the size of the migrating zone, the speed of the separation and the joule heating. The joule heating further influences resolution. Therefore, a high potential gradient for a given current, or heat output, will be preferable.

The above equations can be implemented on simple spread sheet software to determine the physical characteristics of discontinuous buffer systems. A few example buffer systems are shown in **Table 1**. These buffer systems have been designed to vary the trailing ion type and the trailing ion net mobility while keeping the ion speed constant. A Tris-formate buffer, at a formate concentration of 50 mmoL L^{-1}, has been selected as a common leading ion. Tris is used as a common counterion. The ion speed and voltage gradient would be determined by the applied current. These buffer systems exemplify some of the characteristics of discontinuous buffer systems. As can be seen, the trailing ion concentration is lower than the leading ion concentration and a wide range of net mobilities can be achieved when using a common leading ion. Also notice that the net mobility, or actual transport of the trailing ion, is lower than the free mobility when using weak electrolytes. Such buffer systems can be used to assess the stacking limits, or mobility, of the analytes to be separated. Sample ions that migrate in the ion front will have a mobility intermediate between the leading and trailing ions. Those sample ions that have a mobility slower than the net mobility of the trailing ion will migrate more slowly than the ion front and will electrophorese within the trailing zone.

Other examples of calculated buffer systems are shown in **Table 2**. These buffer systems vary the trailing ion type while keeping the ionic strength of the trailing phase constant. This is accomplished by varying the leading ion concentration to allow for a predetermined ion concentration in the trailing zone. All the buffer systems in Table 2 use a formate leading ion of varying concentration and have a common trailing ion concentration of 30 mmol L^{-1}. Even with a common trailing ion concentration, a 50% change in the conductance can be obtained. This allows for increased voltages to be applied without increasing the current. As with the buffer systems described in Table 1, a range of trailing ion net mobilities is obtained. Other buffer systems, that define different ranges of net mobilities, different pH values or different conductivities, could be similarly calculated to address particular separation problems or analytical characterizations.

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Outlook

Electrophoretic techniques that exploit the differential migration of ions are over 100 years old. Though the applications and physical formats have evolved, the underlying technique continues to endure. The iteration of discontinuous buffer systems has had intermittent use since its introduction over 30 years ago. This is presumably due to the perceived added

Table 1 Examples of discontinuous buffer system using constant leading ion conditions[a]

Trailing ion species	m *Mobility* ($\times 10^4$ cm^2 V^{-1} s^{-1})	*Leading ion pH*	*Concentration of trailing ion species* (mmol L^{-1})	*Ion concentration* (mmol L^{-1})	*Net mobility* ($\times 10^4$ cm^2 V^{-1} s^{-1})	κ *Trailing zone conductance* (cm^2 Ω^{-1} mol^{-1})
Hepes	1.45	8.0	26.4	25.1	1.38	0.98
Tricine	2.18	8.0	33.6	27.1	1.76	1.25
Asparagine	2.80	8.5	38.2	23.2	1.70	1.20
Glycylglycine	2.85	8.0	38.5	27.8	2.06	1.47
Taurine	3.27	9.0	41.0	27.2	2.17	1.54
Glycine	3.74	8.0	43.4	9.4	0.81	0.58

[a]The leading ion in all cases is 50 mmoL L^{-1} formate (ion mobility of 5.50×10^{-4} cm^2 V^{-1} s^{-1}) buffered with Tris (ion mobility of 2.60×10^{-4} cm^2 V^{-1} s^{-1}) to the indicated pH. The counterion in all cases is Tris.

Table 2 Examples of discontinuous buffer systems with constant trailing ion concentration[a]

Trailing ion species	*Concentration of trailing ion species* (mmol L^{-1})	*Ion concentration* (mmol L^{-1})	*Net mobility* ($\times 10^4$ cm^2 V^{-1} s^{-1})	*Trailing zone conductance* (cm^2 Ω^{-1} mol^{-1})	*Leading ion concentration* (mmol L^{-1})
Hepes	31.6	30	1.38	1.17	60
Tricine	37.2	30	1.76	1.38	55
Asparagine	49.4	30	1.70	1.56	65
Glycylglycine	41.5	30	2.06	1.58	65
Taurine	45.2	30	2.17	1.70	55
Glycine	138.5	30	0.81	1.83	160

[a] The leading ion in all cases is formate (ion mobility of 5.50×10^{-4} cm^2 V^{-1} s^{-1}) at the indicated ion concentration. The counterion in all cases is Tris (ion mobility of 2.60×10^{-4} cm^2 V^{-1} s^{-1}).

complexity of the technique or ignorance of the technique's advantages. However, the benefits of sample stacking, mobility tailoring and an ionic reference front are unchanged and unique when compared to zonal buffer systems. With new applications and challenges for electrophoretic separations, renewed attention to the technique is certain.

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Electrochromatography

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Introduction

The application of electric fields to planar chromatographic media in order to drive and/or enhance separations is as old as planar chromatography itself. There is evidence to suggest that paper electrophoresis was first performed several years prior to the first reports of paper chromatography. Research in the field has been intermittent, with periods of considerable activity separated by long periods of inactivity. This is at least partly due to attention being diverted away from planar methods to modern high-efficiency column techniques. It is quite conceivable that modern chromatography would be very different, with a much stronger focus on planar techniques, had more development work been carried out on thin layer electrochromatography (TLE).

The subject is enjoying something of a revival with significant advances having been made during the